

Inhibition of Lung Cancer Stem Cell Migration and Growth Through Nano-Photodynamic Therapy

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Abstract. Recurrence and post-treatment spreading of lung cancer signify the existence of drug-resistant cancer stem cells (CSCs) that withstand current therapies. The development of metastases and secondary tumors is a primary contributor to mortality. CSCs play a pivotal role in driving cellular invasion and have a critical impact on prognosis. Enhancing treatment outcomes involves the targeted elimination of CSCs while preserving healthy tissue. In this laboratory-based investigation, Photodynamic Therapy (PDT), a minimally invasive treatment, was employed alongside a nano drug carrier for lung cancer. PDT operates by inducing light-triggered cell death using a photosensitizing drug. When coupled with gold nanoparticles, this nano-mediated PDT facilitated cell death specifically in lung CSCs. The study's objective was to assess the impact on normal lung cells and isolated CSCs, considering aspects such as cellular structure, migratory ability, proliferation, toxicity, doubling time, and the cell cycle. The results revealed minimal effects on normal lung cells, a reduction in CSC migration and invasion, the initiation of cell cycle arrest, and a decrease in CSC proliferation. Utilizing a drug nano carrier like AuNPs significantly enhanced the efficacy of PDT, with a specific focus on curtailing CSC-mediated dissemination in lung cancer, ultimately leading to an improved prognosis. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: gold nano sensitizer; photodynamic therapy; dissemination; lung cancer stem cells.

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1 Introduction

Photodynamic therapy (PDT) is a promising cancer treatment. PDT produces reactive oxygen species (ROS) upon photoactivation of a photosensitizer (PS). Cancer stem cells (CSCs) are inhibited by oxygen-dependent exogenous ROS. PDT may also raise ROS levels by influencing metabolism, endoplasmic reticulum stress, or mitochondrial membrane potential. PDT induced ROS have a short half-life, are highly reactive, and have limited diffusion. Thus, PDT's photodynamic action is seen at the subcellular level, which aids in understanding how PDT affects CSC characteristics such as differentiation, self-renewal, apoptosis, autophagy, and immunogenicity. Excess ROS disrupts the redox system,

oxidizes DNA, alters mitochondrial permeability, and activates the unfolded protein response, autophagy, and the CSC resting state. Thus, understanding the molecular mechanism by which ROS affect CSCs improves PDT and prevents tumor recurrence and dissemination [1]. Gold nanoparticles have gained attention in cancer therapy due to their unique physicochemical properties and potential applications in PDT. Gold nano-sensitizers have been utilized as promising agents in PDT to enhance the efficiency of light-based treatments by amplifying the generation of ROS and improving the therapeutic outcomes [2].

Evaluating the effects of nanoPDT using gold nanoparticles (AuNPs) as drug delivery vehicle on lung CSC proliferation, invasion, and migration is significant

due to the unique characteristics and role of CSCs in cancer progression and treatment resistance. CSCs are a small subset of cells within tumors that possess self-renewal capabilities and have been associated with tumor initiation, relapse, and dissemination [3]. Traditional cancer therapies often target the bulk of tumor cells but may fail to effectively eliminate CSCs, leading to tumor recurrence and treatment failure. Therefore, it is crucial to develop therapeutic strategies that can specifically target and eradicate CSCs to achieve long-term remission and improve patient outcomes.

The current state of research on nanoPDT using AuNPs on lung CSC proliferation, invasion, and migration is still an emerging field with limited studies available, where related research on the topic has only been reviewed. However, related, and preliminary research suggests promising potential for using gold nano-sensitizers in PDT to target and inhibit the behaviors of lung CSCs. Previous research on A549 lung cancer using AlPcS₄Cl and AuNPs indicated that the compound can prevent lung cancer migration and invasion, induce cell cycle arrest and reduce its proliferative abilities [4]. These results only related to lung cancer alone and not the effects of the compound on the side population of CSCs and normal lung cells. Mkhobongo et al. (2022) investigated the effects of PDT on melanoma cells (A375) and their stem cell population using AlPcS₄Cl and AuNPs. The study found that the drug delivery vehicle (AuNPs) increased PS uptake and localization in mitochondria and lysosomes, resulting in a dose-dependent decrease in melanoma viability [5]. Researchers investigated the efficiency of a nanoparticle conjugate loaded with Indocyanine green and Protoporphyrin IX on cobalt ferrite superparamagnetic nanoparticles as a multifunctional compound for nanoPDT in another study on A375 CSCs. The study found that ROS and cell death were effectively increased [6].

These studies provide preliminary evidence supporting the use of nanoPDT for inhibiting CSC proliferation, invasion, and migration. However, further research is required to validate these findings and optimize the treatment protocols. It is important to note that the field of gold nano-sensitizer PDT on lung CSCs is still evolving, and more studies are needed to fully understand its potential and establish its clinical relevance.

By evaluating the effects of nanoPDT on lung CSCs, researchers aim to investigate the potential of this treatment modality in specifically targeting and suppressing the proliferation, invasion, and migration abilities of CSCs. The study intends to shed light on the effectiveness of nanoPDT as a novel therapeutic approach in overcoming CSC-related challenges in lung cancer treatment. Understanding the impact of using AuNPs as PS deliver vehicle during PDT on lung CSCs' behavior is essential for developing personalized and targeted therapies that can eradicate CSCs and prevent cancer recurrence and dissemination. This research may provide valuable insights into the development of

innovative treatment strategies for lung cancer patients, ultimately improving their prognosis and quality of life.

The study assessed the photodynamic effects of gold nano-sensitizer PDT on normal lung epithelium and isolated lung CSCs by evaluating the morphological and biochemical effects on normal lung cells and determining the effects on CSC migration, proliferation, and invasion.

2 Methods

2.1 Cell Culture and CSC Separation

Normal lung epithelial cells (HBEC3-KT (ATCC® CRL-4051™)) were cultured in Airway Epithelial Cell Basal Medium (ATCC PCS-300-030) and Bronchial Epithelial Cell Growth Kit (ATCC PCS-300-040). Cancer cells were cultured in Rosewell Park Memorial Institute 1640 medium (RPMI-1640) (Sigma-Aldrich, R8758) supplemented with 5% fetal bovine serum (FBS) (Biochrom, S0615) and 0.5% penicillin/ streptomycin (Sigma-Aldrich, P4333) and 0.5% amphotericin B (Sigma-Aldrich, A2942). A side population of CSCs were sorted using a magnetic bead isolation kit (Miltenyi Biotec, QuadroMACS™ separation unit 130-091-051) and characterized using flow cytometry. CSCs enriched for the respective markers were maintained at a passage between 4 and 8 for all experimental purposes. All cultures were maintained and incubated at 37 °C in 5% CO₂ and 85% humidity.

2.2 Immunophenotyping of Lung CSCs

The protein expression of the CSC markers CD133, CD44 and CD56 (CD133 Antibody (3F10), NovusBio, NBP2-3774 / CD44 Antibody (8E2F3), NovusBio, NBP1-47386 / CD56 Monoclonal Anti-N Cam, Sigma-Aldrich, C9672) was identified using the secondary antibody identification technique. Suspension cells were labelled with primary human anti-mouse and identified with secondary fluorescent Goat anti-Mouse (NovusBio, NB720-F-1 mg; NovusBio, NB7602; NovusBio, NB7594). Antigenic expression was examined by flow cytometry (BD Accuri Flow Cytometer C6).

2.3 Photodynamic Therapy

The gold nano-sensitizer in this study was synthesized and characterized as described in previous research [7] by loading Al (III) Phthalocyanine Chloride Tetra sulfonic Acid (AlPcS₄Cl) (Frontier Scientific, AlPcS-834) onto gold nanoparticles (AuNPs) (Sigma-Aldrich, 765465). Lung CSCs were cultured and seeded at a total of 5×10^5 cells per petri dish in complete media and allowed to attach for 24 h before irradiation. Cultures were divided into 4 study groups. Group 1 was the control and received no PDT, group 2 received PBM alone and was irradiated at 10 J/cm². Group 3 received PDT treatment with 10 J/cm² photoactivation and 20 µM AlPcS₄Cl and group 4 received the gold nano-sensitizer and 10 J/cm² photoactivation. All samples were incubated for 24 h after PDT treatment followed by

morphological and physiological analysis. For migration studies cells were incubated for 24- and 48-h post irradiation. The concentrations of the sensitizers and laser parameters used are indicated in Table 1.

2.4 Morphology: Inverted Light Microscopy

Morphological differences were observed and examined 24 h after irradiation using an inverted light microscope (OLYMPUS, CKX41). The microscope was equipped with a digital camera (OLYMPUS, SC30) connected to it, allowing the capture of images. The Olympus cellSens Software was utilized for image acquisition and analysis.

2.5 Cell Migration: “Central Scratch Test”
Method

The migration of cells was assessed using the “central scratch” technique. The cells were cultured in 35 mm petri dishes and incubated overnight at 37 °C and 5% CO2. Before irradiation, a central scratch was created using a sterile P-200 pipette tip. The movement of the cells was observed using specific positions and focal planes on an Inverted Microscope (Wirsam, Olympus CKX41) and documented using a digital camera (SC30 Olympus Camera). Images were captured at 0 h, 24 h, and 48 h after irradiation. Velocity (*v*) a vector quantity that measures displacement (or change in position, Δ*s*) over the change in time (Δ*t*) was calculated using the following Eq.: $\bar{v} = \frac{\Delta x}{\Delta t}$, where the distance that the cells travelled was measured and the mean cell velocity (μm/h) tabulated. Area migration was quantified by calculating the area of the image covered by cells using Image J analysis software, which converts the cell population image to a mean grey value and calibrates the area measured using the image’s scale bar.

2.6 Cell Proliferation and Deterioration

The Cell Titer-Glo test (Promega, G7573) was used to detect cell proliferation as a marker of metabolically active cells. The assay produces a bright signal indicating the presence of ATP production in metabolically active or developing cells. The ATP test takes advantage of luciferase’s characteristics to provide a sustained luminous signal while preventing endogenous ATP

release during cell lysis. To induce cell lysis, equal amounts of reconstituted reagent and cell suspension were introduced and stirred. The luminous signal was quantified in Relative Light Units using a luminometer (Perkin Elmer, Victor3) (RLU).

The release of LDH from the cytosol, resulting from induced membrane disruption, was utilized to quantify the deterioration of cells. The CytoTox96® nonradioactive cytotoxicity test (Promega, G400) was employed for this purpose. In this test, LDH interacts with a tetrazolium salt (present in the reconstituted reagent) in its oxidized form, leading to the formation of a measurable red formazan product in its reduced form. This chemical reaction is dependent on NADH. The absorbance of the resulting color was measured spectrophotometrically at 490 nm using a multi-label Counter (Perkin Elmer, VICTOR3™, 1420).

2.7 Cell Viability-Proliferation Kinetics:
Population Doubling Time

To detect live and dead cells, a Trypan Blue Stain (0.4%) assay was performed (Invitrogen™, T10282). This assay relies on the principle of color exclusion. An Automated Cell Counter (ThermoFischer, AMQAX1000) was used to quantify the number of viable cells. The doubling time represents the duration required for a given quantity to double in size or value, assuming a constant growth rate.

To calculate the population doubling time of the samples, the following Eq. was utilized:

$$gr = \frac{\ln(N(t)/N(0))}{t},$$
$$dt = \frac{\ln 2}{gr}.$$

The growth rate was determined by dividing the number of cells by the initial seeding density. Subsequently, the doubling time was calculated as an exponential rate, taking into account the viable cell count at seeding (*N*(0)), the viable cell count at harvest (*N*(*t*)), and the time interval (*t*) in hours between seeding and harvest. The viability of cells was determined using trypan blue exclusion method.

Table 1 Gold nano-sensitizer and irradiation parameters.

PDT Parameters	
AlPcS4Cl Concentration (μM)	20
AuNPs Concentration (ppm)	20
Wavelength (nm)	659.4
Type	LED Well plate illuminator
PSU	Keithley 2200-32-3
Power density (mW/cm²)	117.17
Fluence (J/cm²)	10
Time of irradiation (s)	85.4

2.8 Cell Cycle Analysis: PI

Flow cytometry was utilized for cell cycle analysis, which involved quantifying the DNA content. In this analysis, a stoichiometric dye called PI (propidium iodide) was employed, which binds to DNA in proportion to its amount within the cells. The experimental procedure involved harvesting the cells and washing them with PBS (phosphate-buffered saline). Subsequently, the cells were fixed by treating them with 70% ethanol for 30 min at 4 °C. After two additional washes with PBS, the cells were treated with 50 µl of a 100 µg/ml stock solution of RNase. Following that, the cells were stained with 50 µg/ml of PI.

2.9 Statistical Analysis

Statistical examinations were carried out on datasets sourced from no fewer than three independent experiments. The comparison between two groups was conducted using Student's *t*-test, while simultaneous comparisons among multiple groups were performed using one-way ANOVA. These analyses were executed utilizing SigmaPlot software version 12, which automates mathematical transformations and statistical analyses. It is noteworthy that both Student's *t*-test and one-way ANOVA assume data normality, a condition verified using the Shapiro-Wilk test. Quantitative analysis of migration morphology data was performed using Image J, a publicly available image processing program based on Java (National Institute of Health, Bethesda, MD, USA). The results are presented as the mean ± standard error (SE). Statistical differences were indicated in tables and graphs using the symbols

$P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***), while dispersion bars were used to represent the standard error.

3 Results

3.1 Synthesis and Characterization of NanoPDT

Synthesis and characterization of this work has been already published in 2020 [7]. Synthesis of nanoPDT was done using a centrifugation process. Since the average size of the nanoPDT was 61.99 nm, the composite can still be categorized as a nano-structure because nanostructures are material configurations with a size between 1 and 100 nm. The nanoPDT suspension appeared to be polydisperse with a modest size distribution based on the polydispersity index (PDI) value of 0.477. The AuNP-PEG-COOH showed O-H stretching at 3422 cm⁻¹, which was caused by the compound being suspended in water, C=C stretching at 1642 cm⁻¹, PEG functionalization-related O-H bending at 1413.9–1468.5 cm⁻¹, carboxylic acid-related O-H bending, and C-O stretching at 1100 cm⁻¹ due to the polyether structure in PEG. AlPcS₄Cl-AuNP-PEG-COOH also had O-H stretching at 3422 cm⁻¹, C=C stretching at 1642 cm⁻¹, C-H bending at 1471 cm⁻¹, O-H stretching at 1430 cm⁻¹, and S=O stretching at 1308.8 cm⁻¹ and 1072 cm⁻¹ from the sulphate functionalized PS. AuNP-PEG-COOH and AlPcS₄Cl both have 520 nm and 676 nm absorption spectra, respectively, as shown by ultraviolet-visible spectroscopy (UV-Vis) of the individual molecules.

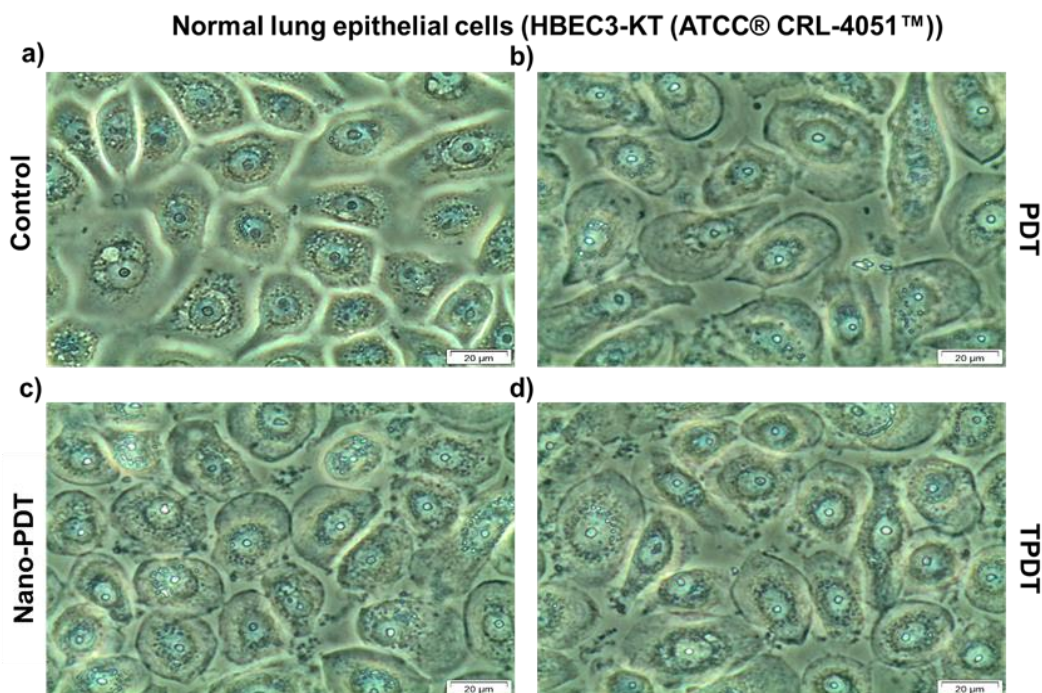


Fig. 1 Morphology of normal lung cells post PDT. Results show normal lung epithelial morphology of the (a) control cells that received no treatment, (b) cells that received PDT treatment, (c) cells that received nanoPDT treatment, and (d) TPDT treated cells, 24 h post PDT treatments.

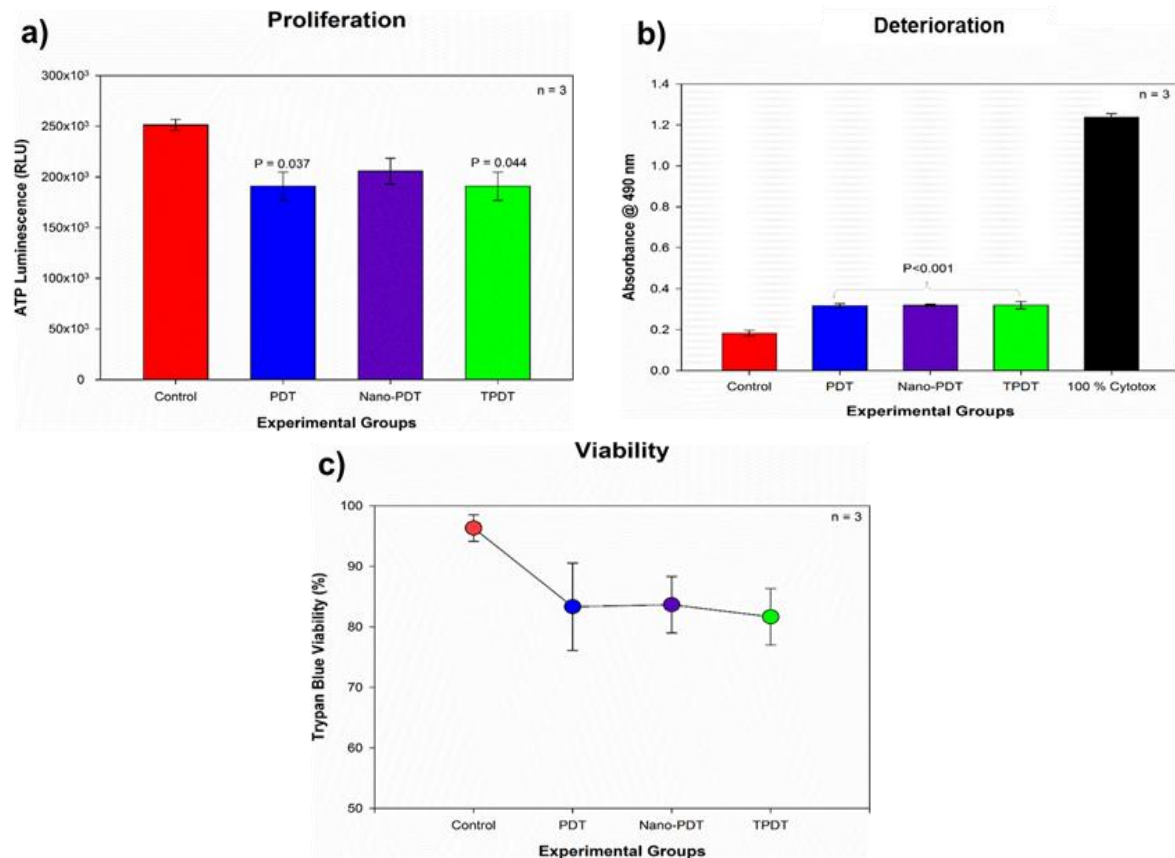


Fig. 2 Biochemical analysis of normal lung cells post PDT treatment. Results show (a) a significant decrease in proliferation of $P = 0.037$ when comparing PDT treated samples to the control and $P = 0.044$ when comparing TPDT treated samples to the control, (b) a significant increase of $P < 0.001$ in cytotoxicity for all PDT treated samples compared to the control and (c) an insignificant decrease in viability post PDT treatments.

The concentration distributions of the AuNPs and the PS in relation to their corresponding dependent absorbance were both linear. Each molecule's linear regression was determined, and the regression coefficients were computed [7].

3.2 Effects of NanoPDT on Normal Lung Cells

3.2.1 Morphology of Normal Lung Cells Post NanoPDT Treatment

The morphology findings (Fig. 1) of normal lung cells at a 24-h interval following photodynamic therapy (PDT) treatments demonstrate that various treatment modalities, including PDT utilizing the photosensitizer (PS) alone with photoactivation, nanoPDT involving PS conjugated to gold nanoparticles, and targeted-PDT (TPDT) incorporating PS conjugated to gold nanoparticles along with an attached antibody, exhibit cellular morphology comparable to that observed in the control experiment with no treatments administered. The observed cellular structures exhibit preserved cell membranes, minimal vacuolization, and a compact monolayer arrangement. There is a lack of substantial alteration in morphology.

3.2.2 Biochemical Examination of Normal Lung Cells Following NanoPDT Treatment

The biochemical findings presented in Fig. 2 indicate a reduction in proliferation among the experimental groups. Specifically, the samples treated with PDT and TPDT demonstrate a statistically significant decrease in proliferation compared to the control group. However, it is important to note that these results are not as significant as the findings presented in Fig. 7(a), which demonstrate a minimum 50% decrease in proliferation among CSCs when subjected to PDT. There is a notable rise in the occurrence of membrane damage, resulting in the leakage of lactate dehydrogenase (LDH) into the surrounding medium across all photodynamic therapy (PDT) groups, as compared to the control group. However, this lack of significance is observed when comparing the results obtained regarding the cytotoxic effects induced in the CSCs (Fig. 7(b)), as well as when comparing it to the positive control, which serves as an indicator of complete cytotoxicity. In a similar vein, the findings pertaining to viability indicate a decline in the percentage of viability. Nevertheless, it is important to note that these results do not exhibit statistical significance. In addition to the morphological findings, the data indicate that the PS exhibits potential in selectively inducing apoptosis in tumor cells while preserving the viability of normal cells.

3.3 Effects of NanoPDT on Lung CSCs

3.3.1 Immunophenotyping of Lung CSCs

Antigenic detection was performed on the separated cells to determine their stem-ness using the BD Accuri™ C6 Flow Cytometer. Indirect antibody labelling enabled the detection of antigen-antibody conjugation fluorescently. Flow cytometry analysis revealed that the separated cells exhibited the markers CD133, CD44, and CD56 (Fig. 3).

3.3.2 Morphology: Inverted Light Microscopy

The morphology of lung CSCs was examined 24 h after PDT treatment. The treated samples were compared to a control sample that did not undergo photoactivation. The results are presented in Fig. 4, which illustrates the appearance of lung CSCs under different conditions. In Fig. 4(a)), untreated lung CSC morphology is shown. In Fig. 4(b)), lung CSCs treated with photobiomodulation (PBM) exhibit no significant changes in morphology compared to the control sample. These results indicate that the cells maintain their viability and ability to proliferate, as evidenced by a dense monolayer of cells. Figs. 4(c) and 4(d) represent samples that underwent PDT and nanoPDT treatment, respectively. In both cases, these samples demonstrate notable morphological alterations compared to the control sample. These changes include cell shrinkage, condensation of chromatin, cancer cell blebbing, cytoplasmic vacuolization, as well as loss of cellular shape, detachment, and the presence of free-floating cells. These observed changes collectively indicate cell death [8], translating into a decrease in cell dissemination as cell death limits the invasion and dissemination of cancer cells [9].

3.3.3 Cell Migration

The evaluation of migration was conducted on the morphological samples. Figs. 5(a) and 5(b) illustrate the

migration patterns of control cells and cells subjected to photobiomodulation (PBM), revealing a similar trend where the cells migrate towards the central scratch. In contrast, Figs. 5(c) and 5(d) demonstrate the effects of PDT and nanoPDT treatment on CSCs. After PDT treatment, the migration rate of the cells decreased, and they were unable to close the scratch over time. This impaired migration can be attributed to cell arrest induced by PDT.

The findings presented in Fig. 6(a) reveal a significant decrease in velocity when comparing the samples treated with PDT and nanoPDT to the control. Table 2 further demonstrates that the mean cell velocity of the PDT and nanoPDT treated samples was significantly lower compared to both the control and PBM samples. Among the treated samples, CSCs treated with nanoPDT exhibited the slowest velocity, although this difference did not reach statistical significance when compared to the samples treated with PDT. In terms of area migration, as depicted in Fig. 6(b), it represents the extent of cell coverage 48 h after irradiation. The results indicate a statistically significant distinction between the PBM, PDT, and nanoPDT treated samples when compared to the control. PBM treatment resulted in a significant increase in area coverage, while both PDT and nanoPDT treatments led to a significant decrease in area coverage. Notably, the two PDT-treated samples displayed notable differences, with nanoPDT showing the greatest reduction in area migration.

Table 2 Mean cell velocity of lung CSCs post irradiation.

	Mean cell velocity (µm/h)
Control	5.96 ± 0.5
PBM	5.16 ± 0.47
PDT	0.22 ± 0.37
nanoPDT	0.08 ± 0.11

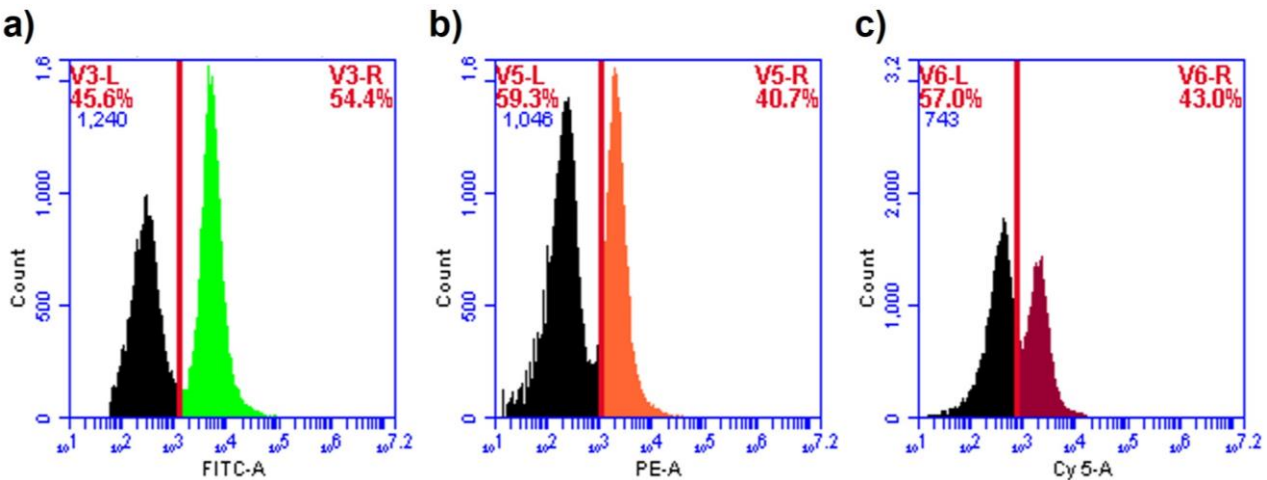


Fig. 3 Flow cytometry expression of stem cell markers (a) CD 133, (b) CD 44, and (c) CD 56 of sorted lung cancer cells.

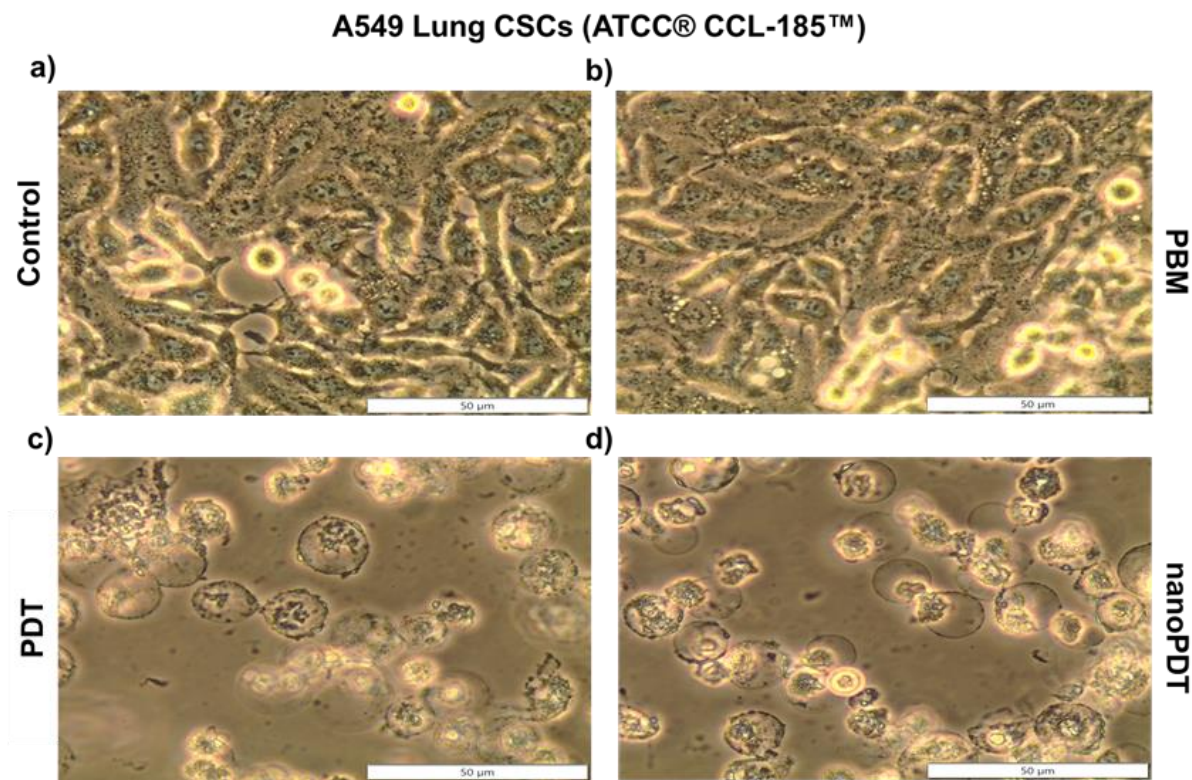


Fig. 4 Morphology of lung CSCs 24 h post PDT treatment. (a) Untreated control cells, (b) CSCs receiving PBM treatment (c) cells receiving PDT treatment, and (d) cells treated using nanoPDT.

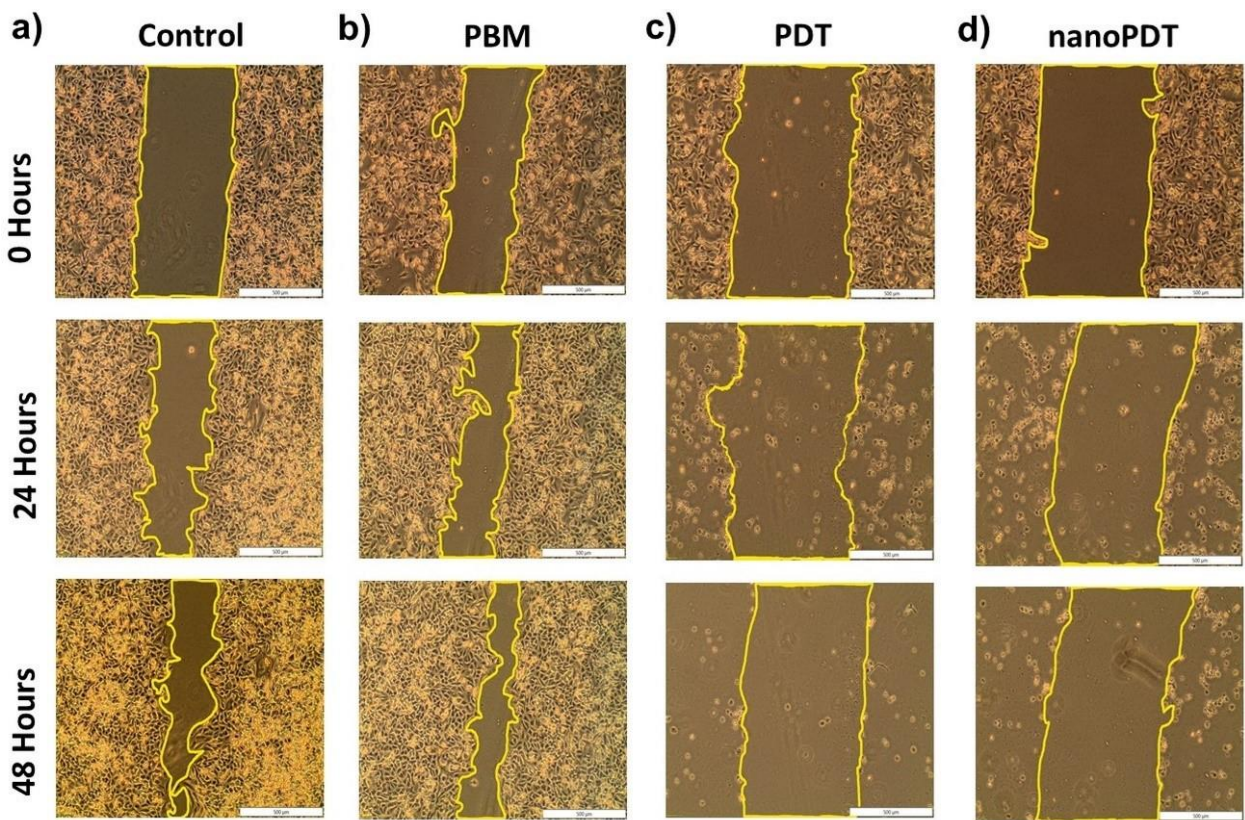


Fig. 5 Morphology of lung CSCs migration 0-, 24- and 48-h post PDT treatment. (a) Control cells that have not received any treatment, (b) CSCs that received only 660 nm PBM at 10 J/cm², (c) cells that received PDT treatment with 20 µM AlPcS₄Cl and 660 nm photoactivation at 10 J/cm², and (d) cells that received nanoPDT treatment with 20 ppm AuNPs and 660 nm photoactivation at 10 J/cm².

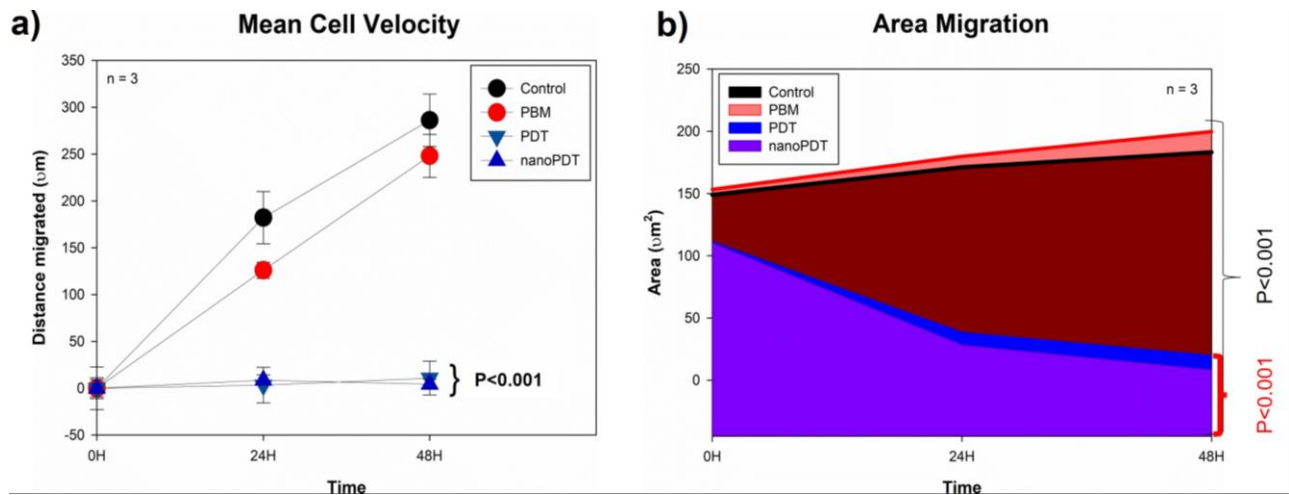


Fig. 6 Lung CSC velocity and area migration 0, 24, and 48 h post irradiation. (a) Mean cell velocity, where PDT and nanoPDT treated samples had a significant ($P < 0.001$) decrease in MCV and (b) area migration, where PBM treated samples had a significant increase ($P < 0.001$) in area migration, and PDT and nanoPDT samples had a significant ($P < 0.001$) decrease.

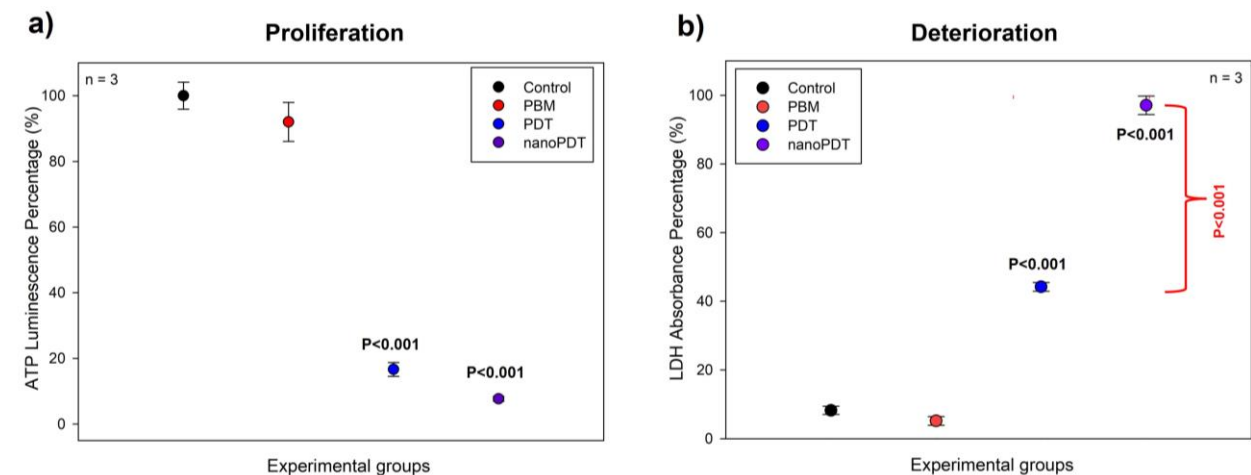


Fig. 7 Lung CSC (a) proliferation, where PDT and nanoPDT had a significant ($P < 0.001$) decrease compared to the control, and (b) deterioration, where PDT and nanoPDT had a significant ($P < 0.001$) increase compared to the control and furthermore a significant increase ($P < 0.001$) was seen in nanoPDT compared to PDT alone, 24 h post irradiation.

3.3.4 Cell Proliferation and Deterioration

The impact of PDT and nanoPDT on lung CSCs was evaluated by measuring intracellular adenosine triphosphate (ATP) levels and LDH release. The quantification of cell proliferation, depicted in Fig. 7(a), was performed by measuring relative light units and converting them into a percentage value using ATP luminescence. The proliferation of CSCs treated with PDT and nanoPDT exhibited a significant reduction compared to both control cells and CSCs treated with PBM alone. Although there was a slight decrease in the number of CSCs treated with nanoPDT compared to PDT, this difference did not reach statistical significance.

The assessment of cell deterioration, presented in Fig. 7(b), was conducted by measuring the absorbance of released LDH resulting from cellular damage and converting it into a percentage value. Control cells and

samples treated with PBM demonstrated minimal LDH release into the surrounding environment. In contrast, CSCs treated with PDT and nanoPDT both exhibited significant LDH release. Notably, it was observed that nanoPDT-induced disruption of the cell membrane led to a significant increase in LDH release when compared to the PDT-treated samples.

The objective of the study was to assess the proliferation and growth rates of lung CSCs before and after irradiation treatment, aiming to determine the effectiveness of PDT in inhibiting growth and inducing cellular degradation. The results, as depicted in Fig. 8, demonstrate that prior to irradiation, all samples exhibited a linear increase in growth rate. However, following irradiation, control and PBM samples displayed a steady increase in growth rate, whereas PDT samples exhibited a significant decrease.

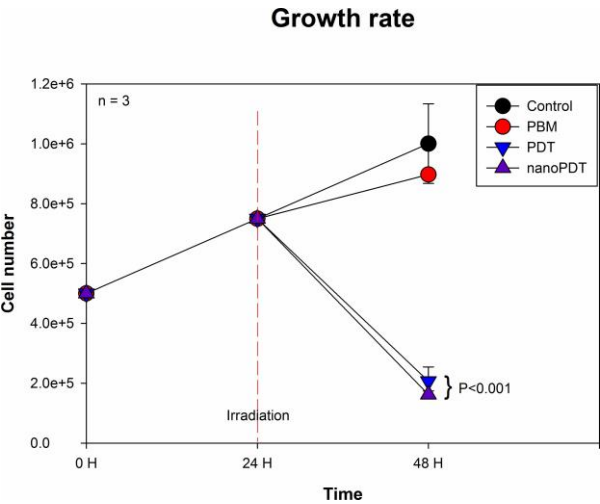


Fig. 8 Growth rate of lung CSCs prior and post PDT treatment. PDT and nanoPDT treated samples showed a significant ($P < 0.001$) decrease in growth rate compared to the control.

Table 3 Population doubling time of lung CSCs 48 h after PDT treatment.

	Ave cell number @ 48 h	Doubling time (h)
Control	1×10^6	47.9 ± 2.82
PBM	8.97×10^5	56.89 ± 3.44
PDT	2.07×10^5	-37.72 ± 3.08
nanoPDT	1.62×10^5	-29.57 ± 3.54

The doubling times, calculated and presented in Table 3, provide insights into the rate at which the cell population would double. For the control and PBM samples, the doubling times indicate that the cell population would double after approximately 48 h and 56 h, respectively. In contrast, the PDT-treated samples exhibited negative doubling times, indicating cellular decay. Specifically, the calculated values suggest that the cell population would be depleted after approximately 37 h for PDT-treated samples and after 29 h for nanoPDT-treated samples. These findings imply that the population of cells treated with nanoPDT would deplete at a faster rate compared to the cells treated with PDT.

3.3.5 Cell Cycle

The cell cycle is a sequential process consisting of four stages that contribute to cell proliferation. These stages include the G1 (Gap 1) phase, where cells mature; the S phase, during which DNA is replicated; the G2 phase, where cells prepare for division and undergo DNA checks; and the M phase, involving mitosis. Cells in the S phase possess more DNA compared to cells in the G1 phase. These S phase cells exhibit a higher uptake of dye and fluorescence until their DNA content has doubled.

Cells in the G2 phase appear approximately twice as bright as cells in the G1 phase. The results presented in Fig. 9 demonstrate that control cells and those treated with PBM predominantly reside in the G1 and S phases. PBM-treated cells show a shift towards the S phase, indicating that PBM stimulates DNA synthesis and cell proliferation. On the other hand, PDT-treated samples in Figs. 9(c) and 9(d) display a significant number of cells entering the G0 phase, resulting in a decreased percentage of cells in G1 and a reduction of cells transitioning out of the S phase. Notably, cells treated with nanoPDT exhibit greater cell cycle arrest compared to PDT-treated samples that were not conjugated to AuNPs.

4 Discussion and Conclusion

In cancer therapy, including PDT, targeting not only the bulk of cancer cells but also CSCs are crucial for achieving long-term treatment success and preventing recurrence. CSCs are a subset of cells within a tumor that are thought to play a role in tumor initiation, growth, dissemination, and resistance to therapy. Because of their unique properties, CSCs are often more resistant to traditional cancer treatments, making them a significant challenge in achieving complete remission. PDT has the potential to target both cancer cells and CSCs while sparing normal cells due to its localized and selective nature [10]. CSCs are highly tolerant to conventional cancer treatments and resilient due to their capacity to migrate and metastasize [11]. Eliminating lung CSCs during therapy is critical because it can prevent CSC proliferation, cancer relapse, and secondary tumor formation. It is consequently critical to know whether a tumor contains a population of cells that exhibit certain stem cell-like characteristics to predict the treatment's prognosis. The isolated cells expressed markers associated with stem cell maintenance, tumor migration and progression, and decreased anti-tumor response. Thus, they are suitable for evaluating drug efficacy, particularly in this case, the enhancement of PDT using AuNPs as a delivery vehicle for the inhibition of lung CSC proliferation and migration. The utilization and investigation of gold nanoparticles as drug delivery vehicles in the context of PDT have been explored. Although gold nanoparticles do not possess inherent photodynamic activity, they can function as vehicles for photosensitizing chemicals [7].

NanoPDT bears promise as a possible strategy to targeting lung cancer stem cells, which are a subpopulation of cancer cells capable of self-renewal and tumor formation. It is critical to target lung cancer stem cells since they are assumed to be responsible for tumor initiation, development, dissemination, and resistance to conventional therapy [12]. Nanoparticles utilized in nanoPDT can be functionalized with specific ligands or antibodies, allowing them to actively target surface indicators or receptors overexpressed on lung cancer stem cells. These ligands or antibodies can promote the selective binding of nanoparticles to lung cancer stem cells, improving photosensitizer uptake and

retention [13]. Active targeting techniques allow for more effective and selective elimination of lung cancer stem cells while causing the least amount of harm to healthy cells [14]. The enhanced permeability and retention (EPR) effect is a feature of tumor tissues, particularly lung cancer stem cells, in which leaky blood arteries and decreased lymphatic outflow lead to increased nanoparticle accumulation in the tumor microenvironment. After intravenous delivery, this action permits nanoparticles to passively collect in tumor tissues [15]. NanoPDT can selectively distribute photosensitizers to the tumor and target lung cancer stem cells within the tumor mass by utilizing the EPR effect [16]. Nanoparticle size and surface charge can alter cellular absorption and dispersion within tumor tissues. Furthermore, surface charge can influence nanoparticle interactions with cancer cells [17]. NanoPDT may be adjusted to increase its' specificity for targeting lung cancer stem cells by carefully manipulating the size and surface features of nanoparticles [7]. Additionally, nanoPDT can be coupled with other stem cell-targeted therapies to improve therapeutic specificity and effectiveness. Therapies that target signaling pathways or molecular markers associated with lung cancer stem cells can be combined with nanoPDT to create a synergistic impact that results in a more successful lung CSC

elimination [18]. However, nanoPDT for targeting lung CSCs is still in the early stages of development. While preclinical investigations have yielded encouraging findings, more research, including in vitro, in vivo, and clinical trials, is required to evaluate the selectivity and therapeutic efficacy of nanoPDT for lung CSCs.

Because most cancer fatalities are caused by metastases, the greatest significant gains in morbidity and mortality will come from preventing or eliminating such disseminated illness [19]. As a result, preventing dissemination is critical for improving patient prognosis. We examined the impact of PDT and its enhanced form utilizing AuNPs as a drug delivery vehicle on lung CSC dissemination. To begin, we examined the migratory impacts of PDT and nanoPDT. Both types of PDT decreased lung CSCs' migratory and invasion potential. NanoPDT significantly decreased cell migration more than PDT alone. It has been proven that PDT can decrease lung cancer dissemination [20]. Additionally, new research indicates that AuNPs can inhibit cancer cell migration/invasion and reduce metastases [21]. Thus, it is hypothesized that combining PDT with nanoparticles would have a greater effect on reducing lung cancer and CSC migration, as demonstrated in this study by a decline in mean cell velocity and a significant reduction in area migration when comparing PDT to nanoPDT.

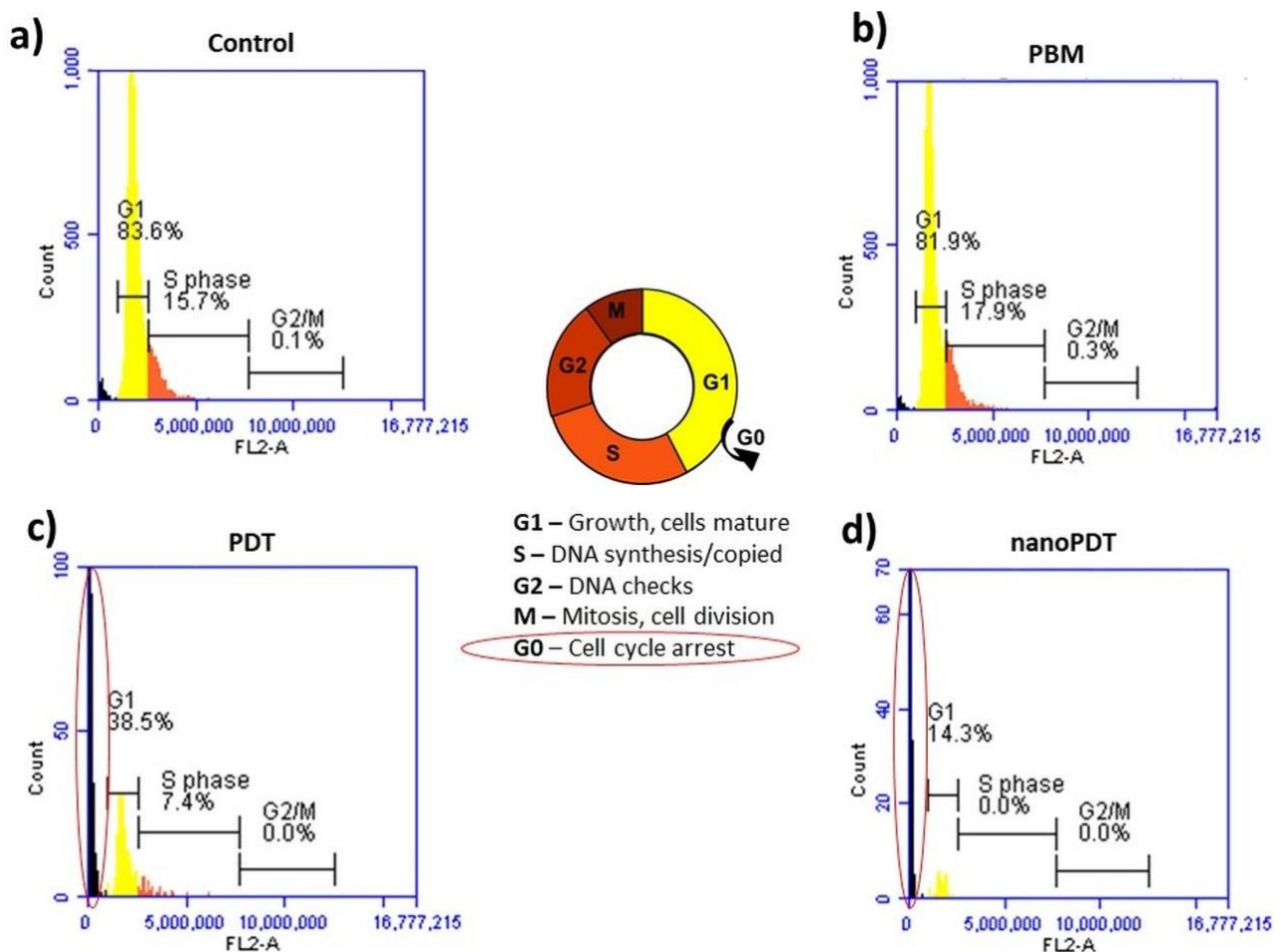


Fig. 9 Cell cycle analysis of CSCs 24 h post PDT treatment. (a) Control cells receiving no treatment. (b) Cells receiving PBM treatment. (c) Cells receiving PDT treatment. (d) Cells receiving nanoPDT treatment.

The amount of ATP in cells can be utilized as a marker for proliferation [22]. Lung cancer cell metabolism is regulated by their mitochondria via aerobic glycolysis in ATP synthesis [23]. Furthermore cancer cell energy metabolism is associated with tumor proliferation and migration [24]. This correlation implies that the altering of lung cancer metabolism through its mitochondrial activity is proportional to lung cancer migration [25], where decreased ATP production is indicative of decreased cell dissemination. Damaged or compromised membranes release the enzyme LDH into the surrounding extracellular space. The quantified amount of LDH is then proportional to the amount of deteriorating cells [26]. CSCs' drug resistance and metastatic potential are a result of their improved capacity to withstand physical stressors that typically result in cell membrane destruction. As a result, circumventing the cell's ability to mend its membrane may result in metastatic inhibition [27]. Effective cytotoxicity can lead to cell regression and death caused by a loss of plasma/cellular membrane integrity or cytoplasmic shrinking. The mechanism through which PDT is harmful is by photosensitized oxidation of biomolecules. Protein and membrane damage is critical for optimizing the cytotoxic efficacy of PDT. Indeed, PSs that accumulate more readily in cell and/or organelle membranes are typically more cytotoxic [28]. PDT has proven successful in decreasing CSC proliferation and increasing membrane damage [7, 29–32], we had previously determined the effects of AlPcS4Cl – PDT and AlPcS4Cl – nanoPDT on lung CSC proliferation and cytotoxicity [7], we replicated these experiments in this study to evaluate the two methods of PDT, statistically demonstrating that nanoPDT improved the efficacy of PDT in causing anti-proliferative and metastatic abilities in lung CSCs.

The rate of cell proliferation is a measure of mitosis, and rapid cancer cell division indicates tumor progression and aggressiveness. Proliferation kinetics is frequently utilized for prognosis and can be used to measure the therapeutic effects of various treatment methods [33] such as PDT. Effective treatment of metastatic cancer frequently necessitates the treatment of both primary tumors and systemic disease undetectable at the time of diagnosis. PDT studies have shown to control primary and metastatic tumor growth and enhances anti-tumor immunity [34]. These outcomes are of importance when considering the abilities of CSCs to metastasize and cause tumor progression. Results indicate that nanoPDT can induce cell cycle arrest in CSCs, disrupting their proliferation and potentially leading to cell death. This arrest can be achieved by the following mechanisms, the PS photoactivation generating ROS causing cellular damage, where the ROS initiate a cascade of cellular responses, including DNA damage, lipid peroxidation, and protein oxidation, which can trigger signaling pathways involved in cell cycle regulation [35]. The damage inflicted by ROS activates cell cycle checkpoints, such as the DNA damage checkpoint and the G2/M checkpoint. These checkpoints

serve as surveillance mechanisms that temporarily halt the cell cycle to allow time for DNA repair and prevent the transmission of damaged DNA to daughter cells [36]. One key regulator of cell cycle arrest is the tumor suppressor protein p53. Activated in response to DNA damage, p53 can upregulate the expression of p21, a cyclin-dependent kinase inhibitor. Elevated p21 levels inhibit cyclin-dependent kinase activity, specifically CDK2 and CDK4, which are essential for cell cycle progression [37]. The activation of cell cycle checkpoints and the induction of p21 can lead to G1 and G2 phase arrest, respectively. G1 arrest prevents damaged cells from entering the S phase, where DNA replication occurs. G2 arrest prevents cells from progressing to mitosis [38]. Depending on the extent of damage, arrested CSCs may either undergo cellular senescence (a state of irreversible growth arrest) or apoptosis (programmed cell death) [39]. These outcomes contribute to limiting the proliferation and survival of CSCs.

The results of this study indicate that PDT have the potential to inhibit tumor progression and dissemination by slowing CSC development, with greater effects observed when nanoPDT is used. The exact mechanism as to why PDT or nanoPDT affect CSCs differently than they do normal cells is unclear. However, the difference in how PDT affects CSCs and normal cells is primarily due to the unique properties and vulnerabilities of CSCs compared to normal cells. CSCs and normal cells may have different rates of photosensitizer uptake due to variations in their metabolic activities and expression of surface receptors. CSCs' distinct properties, such as altered signaling pathways and stem cell markers, can influence their ability to take up photosensitizing agents differently from normal cells. This differential uptake can lead to preferential accumulation of PSs within CSCs, making them more susceptible to PDT-induced damage [29]. CSCs and normal cells may have variations in DNA repair mechanisms and cellular survival pathways. PDT has the potential to target the self-renewal pathway of CSCs indirectly by inducing oxidative stress and cellular damage. While PDT itself does not directly target specific molecular pathways, its effects on cellular processes can disrupt the self-renewal capabilities of CSCs, which are often driven by specific signaling pathways [40]. PDT can trigger an immune response by inducing inflammation and cell death. Immune cells can recognize and target CSCs, further inhibiting their self-renewal capacity [41]. Cancer cells often have higher metabolic rates and more active cellular processes than normal cells. As a result, they may take up photosensitizing agents more readily [42]. Normal cells possess efficient DNA repair mechanisms [43] that may help them recover from DNA damage caused by PDT.

The *in vitro* results obtained indicate a difference in use of PDT versus nanoPDT for CSC destruction. This is of importance when considering *in vivo* and clinical application where the use of PDT can be enhanced by using nanoparticles. The drug delivery capabilities of the

system are highly precise, exhibiting a high level of accuracy. Additionally, the system demonstrates a proficient and effective reaction to visual stimuli, as well as a notable resistance to hypoxia. Additionally, the utilization of nano-photosensitizers (nano-PSs) has been explored to boost the effectiveness of photodynamic therapy (PDT) by increasing the production of ROS. Furthermore, the utilization of nano-sized polymer spheres (nano-PSs) in combination with additive or synergistic medicines has great importance in both ongoing preclinical research and potential future clinical applications. This is due to their capacity to enhance therapeutic efficacy to a significant extent while minimizing the risk associated with systemic drug administration [44]. Furthermore the use of AuNPs have been found to be biocompatible and comparatively less toxic [45], where AuNPs accumulate preferentially in organs like the liver, lung and spleen, where accumulation had been seen to be size dependent [46]. The reticuloendothelial system (RES) plays a key role in the excretion of gold nanoparticles. RES, composed of specialized cells in the liver and spleen, recognizes and engulfs foreign particles, including gold nanoparticles, from the bloodstream. Once engulfed, these nanoparticles become sequestered within RES cells [47]. Over time, the engulfed gold nanoparticles undergo processes such as degradation or transformation. Depending on the nanoparticle's properties, degradation products or transformed forms may be released from the RES cells into the bloodstream. Subsequently, these altered nanoparticles or degradation products can be eliminated from the body through routes like urine or feces. The RES-mediated excretion of gold nanoparticles is a crucial aspect of their pharmacokinetics and biocompatibility, impacting their potential use in biomedical applications [48]. This study showed that PDT directly affects lung CSC viability and proliferation [29], and so indirectly its metastatic abilities of migration and growth rate, where AuNPs significantly enhanced the PDT effects, similarly results indicated that PDT treatment effectivity was improved using nanomaterials [49, 50].

Future perspectives and limitations of this study include that the findings shed light on promising avenues for future research in cancer therapy, particularly in the context of targeting CSCs using PDT and its enhanced form with nanoparticles (nanoPDT). Further exploration is warranted to elucidate the exact mechanisms underlying the differential effects of PDT and nanoPDT on CSCs compared to normal cells. Understanding these mechanisms will be crucial for optimizing treatment strategies and enhancing therapeutic outcomes. Additionally, investigating the use of specific ligands or antibodies for active targeting of nanoparticles to CSCs could improve the selectivity and efficacy of nanoPDT. Moreover, the combination of nanoPDT with other stem cell-targeted therapies holds promise for synergistically inhibiting CSC proliferation and metastasis. However, it's essential to recognize that nanoPDT for targeting CSCs is still in its early stages of development, and further

research, including preclinical and clinical trials, is necessary to validate its selectivity and therapeutic efficacy. Despite the promising findings, several limitations should be considered when interpreting the results of this study. Firstly, the research primarily relied on *in vitro* experiments, which may not fully recapitulate the complex tumor microenvironment and interactions between different cell types observed *in vivo*. Therefore, future studies should incorporate *in vivo* models to better assess the efficacy and safety of nanoPDT for targeting CSCs in a more physiologically relevant setting. Additionally, while the use of AuNPs as drug delivery vehicles shows potential advantages, such as biocompatibility and low toxicity, their pharmacokinetics and biodistribution need further investigation to ensure their safe and effective utilization in clinical applications. Moreover, the study focused on lung CSCs, and extrapolating these findings to other types of CSCs or cancers may require additional validation. Finally, while PDT and nanoPDT demonstrated promising results in inhibiting CSC proliferation and migration, their long-term effects on tumor recurrence and patient survival remain to be evaluated through rigorous clinical trials. Therefore, caution should be exercised when translating these findings into clinical practice until further evidence is gathered.

In conclusion, the primary goal of photodynamic treatment (PDT) is to selectively eliminate cancer cells while preserving healthy cells. The results of this study demonstrate that the application of PDT to normal lung cells led to some toxicity and reduced growth. However, these effects were less pronounced compared to the impact on cancer cells, which were completely eradicated. Importantly, normal lung cells maintained favorable morphological characteristics and exhibited high viability despite exposure to PDT. Considering the role of cancer stem cells (CSCs) in cancer recurrence and dissemination, it is crucial for cancer treatments to effectively target and suppress their abilities. The results of PDT and nanoPDT on the proliferation and migration of CSCs revealed significant reductions in cell migration, velocity, population doubling time, and cell cycle progression. Notably, nanoPDT demonstrated enhanced effects, suggesting a substantial inhibition of CSC mobility. However, before suggesting nanoPDT as a therapeutic treatment for cancer, the current findings must be verified utilizing pre-clinical animal and human clinical investigations.

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Disclosures

The authors declare that they have no conflict of interest.

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